Docket No. 600-1-291CON

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS: Albert et al. EXAMINER: Nickol, Gary B.

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TITLE: METHODS FOR USE OF APOPTOTIC CELLS TO DELIVER ANTIGEN TO DENDRITIC CELLS FOR INDUCTION OR TOLERIZATION OF T CELLS

CERTIFICATE OF MAILING UNDER 37 CFR 1.8

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on July 22, 2005.

Loretta Kavanagh
(Name of person Depositing Mail)

Loretta Kavanagh 7/22/05
(Signature and Date)

DECLARATION PURSUANT TO 37 C.F.R. § 1.132 OF MATTHEW ALBERT, M.D., PH.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Matthew Albert, M.D., Ph.D. do hereby declare as follows:

1. I am a Faculty member in the Department of Immunology at Institut Pasteur, Paris having received my Ph.D. degree in Immunobiology from The Rockefeller University in 1999 and my M.D. degree from Cornell University Medical College in 2000. After that I was a postdoctoral fellow at The Rockefeller University while completing a Clinical Pathology Residency at The New York Presbyterian Hospital.

2. My full curriculum vitae is attached hereto as Exhibit A.

3. My principal area of research is Dendritic Cell Immunobiology and Tumor Immunity, and among other positions I serve as a reviewer in numerous scientific journals including *Science, Nature Immunology, Immunity, PNAS.*

4. I have reviewed the disclosure of the present application, entitled "METHODS FOR USE OF APOPTOTIC CELLS TO DELIVER ANTIGEN TO DENDRITIC CELLS FOR INDUCTION OR TOLERIZATION OF T CELLS" and have also reviewed a reference by Engleman et al. WO94/02156, entitled METHODS FOR USING DENDRITIC CELLS TO ACTIVATE T CELLS. My laboratory has conducted work in this area of research for many years. The means by which Engleman et al. activate T cells is different from the means for activating T cells presented in the current patent application.

5. More importantly, the work presented by Engleman et al. does not take into account the fact that there is a distinct difference between irradiating cells with the intent of sterilizing them for human use (killing adventitial adgents), irradiating cells to make the cells necrotic, as compared to irradiating cells to induce apoptosis. There are distinct advantages of cross-presenting antigen in the context of an apoptotic cell, whereas the same does not hold true for use of a necrotic cell. This is clearly pointed out in the present patent application on page 46, lines 23-33, continuing on to page 47, lines 0-7.

6. Apoptosis is a live, active cell process. Our laboratory has demonstrated that active proteolysis is necessary in the apoptotic cell to allow exogenous antigen cross-presentation (see the enclosed pre-print of a paper entitled "Apoptotic Cells Deliver Processed Antigen to Dendritic Cells for Cross-Presentation" in PLoS Biology, June 2005, Volume 3, submitted herewith as Exhibit B). This obviously would not work for necrotic cells. Furthermore, apoptosis inhibitors, such as Z-VAD, block T cell activation even in the presence of UV irradiation (see the present patent application on page 46, lines 4-22). In addition, my research has demonstrated that only specific forms of death activate T cells, that is, apoptosis, but not necrosis.

7. Without providing the type of irradiation and the exact doses of irradiation (gamma or otherwise), it would be difficult, if not impossible, to predict whether one is inducing apoptosis or necrosis. The present patent application clearly defines those conditions, and furthermore, provides proof that the cells were apoptotic. These conditions are

clearly missing from the Engleman et al. reference. One may assume that Engleman et al. were using gamma irradiation, since it is well known in the field that gamma irradiation induces necrotic death under many conditions, but there is no teaching of this in the Engleman et al. publication. Thus, Engleman et al. do not teach whether they induce necrosis or apoptosis.

8. Engleman et al. could not have known to titrate the exact doses of irradiation for the purpose of inducing apoptosis, rather than necrosis for presenting antigen to dendritic cells. It was not until the work of the present patent application that such specific conditions were made known.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18 of the U.S. Code, Section 1001, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Dated: May 12, 2005

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Diplomas

Brown University, Providence, RI	Sc.B.	1992	Chemistry
The Rockefeller University, New York, NY	Ph.D.	1999	Immunology
Cornell University Medical College, New York, NY	M.D.	2000	Medicine

Honors and Awards

American Chemical Society Award for Top Chemist at Brown University, 1992.

Magna Cum Laude, Brown University, 1992.

Elected as an Associate Member of the Brown University Chapter of Sigma Xi, 1992.

Medical Student Achievement Award. American College of Rheumatology, 1998.

Sumi Koide Fellowship. The Rockefeller University, 1999.

Kean Fellowship for International Medical Research. Cornell University Medical College, 1999.

Distinguished Dissertation Award, Biology and Life Sciences. Council of Graduate Schools, 1999.

The Gustavo Cudkowicz Memorial Prize for excellence in Immunobiology and Biomedical Research. Cornell University Medical College, 2000.

Young Investigator Award. International Congress on Dendritic Cells, 2000.

Rising Star Award. Poly Prep CDS, 2000.

Burroughs Wellcome Career Award in Biomedical Science, 2001.

Regional Winner for North America for the Amersham Pharmacia Biotech & *Science* Prize, 2001.

Paul E. Strandjord Young Investigator Award, Academy of Clinical Laboratory Physicians & Scientists, 2002.

Doris Duke Clinical Scientist Development Award, 2002.

Professional Associations

Sigma Xi, elected member (1992).

American Association of the Advancement of Science, member (since 1993).

Society for Leukocyte Biology, member (since 2000).

Society for Biological Therapy (since 2002).

Academy of Clinical Laboratory Physicians and Scientists (since 2002).

French Society of Immunology (since 2004).

Research Experience and Academic Appointments

1988 – 1992	Undergraduate Research, Solid State Chemistry, Brown University, Providence, RI. Advisor: Dr. Aaron Wold. *Designed materials for use in photovoltaic cells.*
1992 – 1993	Research Technologist, Lawrence Berkeley Laboratories, Berkeley, CA. *Assisted in the development of detection systems specific for environmental toxins.*
1993 – 2000	Biomedical Fellow, Tri-Institutional M.D./Ph.D. Program, New York City, NY. Advisors: Drs. Nina Bhardwaj and Robert Darnell. *Investigated the mechanism of cross-priming T cells specific for tumor antigen.*
1998 – 2000	Guest Investigator, Clinical study, Anandaban Hospital, Nepal. Principle Investigator: Dr. Gilla Kaplan. *Studying the effect of Thalidomide in patients with Erythema Nodosum Leprosum.*
1999 – 2000	Post-doctoral Student, Basic and Clinical Studies, The Rockefeller University. Principle Investigator: Dr. Robert Darnell, *Assisted in establishing a laboratory for studying tumor immunity and autoimmunity; Defined a critic role for $\alpha_v\beta_5$ in the phagocytosis of apoptotic cells.*
2000 – 2003	Post-doctoral Fellow, Basic and Clinical Studies, The Rockefeller University. Principle Investigator: Dr. Robert Darnell. *Investigating the mechanism of naturally occurring tumor immunity & tumor-mediated immunosuppression.*
2000 - 2003	Clinical Scholar, The Rockefeller University. *Pre-clinical research & clinical trials for use of apoptotic cells as a tumor vaccine.*
2001 - 2003	Resident, Clinical Pathology / Laboratory Medicine, The New York Hospital.
2003 - present	Adjunct Faculty, The Rockefeller University. *Co-investigator on clinical trial for use of apoptotic cells as a tumor vaccine in the treatment of Prostate Cancer patients.*
2003 - present	DR2, INSERM and Head of Lab, Institut Pasteur, Paris. *Heading a 7 person group in the area of dendritic cell immunobiology and tumor immunity.*

Active Clinical Protocols

Co-Principle Investigator. The Necker Hospital. Title of study: Effective Tumor Immunity in Transitional Cell Carcinoma of the Bladder (PI: Nicolas THIOUNN).

Co-Principle Investigator. The Necker Hospital. Title of study: Hepatitis C virus pathogenesis and dendritic cell biology (PI: Stanislas POL).

Investigator. The Rockefeller University Hospital IRB#RDA-0466-0103. Title of study: A phase I/II study to evaluate the safety and immunogenicity of the subcutaneous administration of autologous DCs pulsed with apoptotic LNCaP prostate tumor cells in prostate cancer patients. (PI: Robert Darnell)

Original Articles

Joe DeCarlo, **M. Albert**, R. Kershaw, K. Dwight and Aaron Wold. Preparation and Characterization of Iron Substituted II-VI Chalcogenides. *Journal of Solid State Chemistry.* **87**, 443 (1990).

Matthew Albert, Robert Kershaw, Kirby Dwight and Aaron Wold. Preparation and Characterization of Semiconducting $MoTe_2$ Single Crystals. *Journal of Solid State Communications,* **81** (1992).

Matthew L. Albert, Y-M. Gao, Dan Toft, Kirby Dwight and Aaron Wold. Improvement of Photocatalytic Activity of Titanium (IV) Oxide by Photodecompostion of Au on TiO_2. *Materials Research Bulletin.* (1992).

Armin Bender, **Matthew L. Albert**, Anita Reddy and Nina Bhardwaj. The distinctive features of influenza virus infection of dendritic cells. *Immunobiology,* **198**:64-79 (1997).

Matthew L. Albert, Birthe Sauter and Nina Bhardwaj. Dendritic Cells Acquire Antigen From Apoptotic Cells and Induce Class I-Restricted CTLs. *Nature*, **392**:86-89 (1998).

Patrick A. J. Hasslet, L. G. Corral, **M. L. Albert**, and Gilla Kaplan. Thalidomide Costimulates Primary Human T Lymphocytes, Preferentially Inducing Proliferation, Cytokine Production, and Cytotoxic Responses in the $CD8^+$ Subset. *Journal of Experimental Medicine*, **187**:1885-1892 (1998).

Matthew L. Albert, Loise Francisco, Birthe Sauter and Nina Bhardwaj. Immature dendritic cells phagocytose apoptotic cells; a novel role for the $\alpha_v\beta_5$ integrin. *Journal of Experimental Medicine*. **188**: 1359-1368 (1998).

Kayo Inaba, S. Turley, F. Yamaide, T. Iyoda, M. Pack, K. Mahnke, B. Sauter, **M. Albert**, M. Subklewe, D. Sheff, N. Bhardwaj, I. Mellman, and Ralph M. Steinman. Efficient presentation of phagocytosed cellular fragments on MHC class II products of dendritic cells. *Journal of Experimental Medicine*. **188**:2163-2172 (1998).

Matthew L. Albert, Jennifer C. Darnell, Armin Bender, Loise M. Francisco, Nina Bhardwaj and Robert B. Darnell. Tumor-Specific Killer Cells in Paraneoplastic Cerebellar Degeneration. *Nature Medicine*. **4**: 1321-1324 (1998).

Matthew L. Albert, Lisa M. Austin and Robert B. Darnell. Detection and Treatment of Activated T cells in the Cerebrospinal Fluid of Patients with Paraneoplastic Cerebellar Degeneration. *Ann of Neuro*. **48**: 9-17 (2000).

Robert B. Darnell and **Matthew L. Albert**. cdr2-specific CTLs are detected in the blood of all patients with paraneoplastic cerebellar degeneration analyzed. *Annals of Neurology*. **48**:270-271 (2000).

Birthe Sauter, **Matthew L. Albert**, Loise Francisco, Marie Larsson and Nina Bhardwaj. The Cross-priming of Antigen Derived from Apoptotic Cells requires a signal for Dendritic Cell Maturation. *J Exp Med*. **191**: 423-33 (2000).

Jennifer C. Darnell, **Matthew L. Albert** and Robert B. Darnell. cdr2, a Target Antigen of Naturally Occurring Human Tumor Immunity, Is Widely Expressed in Gynecological Tumors. *Cancer Research*. **60**: 2136-2139 (2000).

Matthew L. Albert, Jong-Il Kim, Raymond B. Birge. The $\alpha_v\beta_5$ integrin recruits the Crk/Dock180 molecular complex for phagocytosis of apoptotic cells. *Nature Cell Biology*. **2**: 899-905 (2000).

Matthew L. Albert, Mithila Jegathesan and Robert B. Darnell. Dendritic cell maturation is required for the cross-tolerance of $CD8^+$ T cells. *Nature Immunology*. **2**: 1010-17 (2001). [commentary by Ken Shortman and William R. Heath. Immunity or tolerance? That is the question for dendritic cells. *Nature Immunololgy*. **2**: 988-9 (2001)]

Randy S. Longman, Andrew H. Talal, Ira M. Jacobson, **Matthew L. Albert** and Charles M. Rice. Patients chronically infected with Hepatitis C Virus have functional Dendritic cells. *Blood* **103**: 1026-29 (2004).

Shin Akakura, Sukhwinder Singh, Matthew Spataro, Weiko Akakura, Jong-Il Kim, **Matthew L. Albert** and Raymond B. Birge. The opsonin MFG-E8 is a ligand for the $\alpha_v\beta_5$ integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells. *Experimental Cell Research*, **292**: 403-16 (2004).

Dana E. Orange, Mithila Jegathesan, Howard Scher, **Matthew L. Albert** and Robert B. Darnell. Prostate Cancer Patient Dendritic Cells Effectively Cross Present Tumor Antigen for the Activation of Antigen Specific T Cells: Implications for clinical studies in immunotherapy of prostate cancer. *Prostate Cancer and Prostatic Diseases*, 7: 63-72 (2004).

Nathalie Blachère, Robert B. Darnell and **Matthew L. Albert**. Apoptotic cells deliver processed antigen to dendritic cells for cross-presentation. *PLoS Biology* (in press, 2005).

Randy S. Longman, Andrew H. Talal, Ira M. Jacobson, Charles M. Rice and **Matthew L. Albert**. Normal functional capacity in circulating conventional and plasmacytoid dendritic cells in patients with chronic hepatitis C virus. *Journal of Infectious Disease* (in press, 2005).

Mithila Jegathesan, Robert B. Darnell and **Matthew L. Albert**. A Novel Immunosuppressive Effect of FK506: Skewing the cross-presentation of antigen towards tolerance. *Submitted.*

Deborah Braun, Randy S. Longman and **Matthew L. Albert**. Prostaglandin-E2 regulates the expression and activity of the immunosuppressive enzyme indolamine 2,3 dioxygenase in myeloid dendritic cells. *Blood (in press, 2005).*

Invited Reviews & Book Chapters

Matthew L. Albert and Nina Bhardwaj. Resurrecting the Dead: Dendritic Cells Acquire Antigen from Apoptotic Cells. *The Immunologist,* **6**:194-198 (1998).

Matthew L. Albert, Shannon Turley, Wendy Garret, Ira Melman, Kayo Inaba, Nina Bhardwaj and Ralph M. Steinman. Uptake and Presentation of Phagocytosed Antigens by Dendritic Cells. In *Advances in Cell and Molecular Biology of Membranes and Organelles.* Vol. 5, pp. 361-76 (1999).

Matthew L. Albert. Phagocytosis of apoptotic cells. In *Dendritic Cells.* Vol. 2. In *Dendritic Cells: Biology and Clinical Applications.* Vol. 2, pp. 627-44 (2001).

Matthew L. Albert and Robert Darnell Paraneoplastic neurological degenerations: keys to tumour immunity. *Nature Reviews Cancer.* **4**: 36-44 (2004).

Matthew L. Albert. Death-defying immunity: do apoptotic cells influence antigen processing and presentation. *Nature Reviews Immunology,* **4**: 223-31 (2004).

Deborah Braun and **Matthew L. Albert.** Monitoring Cell Death. In *Measuring Immunity: Basic Biology and Clinical Assessment.* (in press, 2005).

Movie Reviews

Matthew L. Albert. Danger in Wonderland. *Science,* **303:** 1141 (2004).

Selected Abstracts (chosen for oral presentation)

Matthew L. Albert, Birthe Sauter and Nina Bhardwaj. Dendritic Cells but not Macrophages Acquire Antigen From Apoptotic Cells and Induce Class I-Restricted CTLs. The Keystone Meeting on Dendritic Cell Biology. Sante Fe, NM (1998).

Matthew L. Albert, Jennifer C. Darnell, Armin Bender, Loise M. Francisco, Lisa Austin, Nina Bhardwaj and Robert B. Darnell. Tumor-specific and antineuronal killer cells in paraneoplastic cerebellar degeneration. *Annals of Neurology.* **44**, 3 (1998). Presented by R. Darnell at The American Neurologic Association. Toronto, Canada.

Matthew L. Albert and Nina Bhardwaj. Resurrecting the Dead: DCs cross-present antigen derived from apoptotic cells on MHC I. The Workshop on Myeloid Cells and their interactions with Lymphoid Cells. Savannah, GA (1998).

Matthew L. Albert and Nina Bhardwaj. Immature DCs phagocytose apoptotic cells and cross-present antigen on MHC I. *Arthritis and Rheumatism.* Suppl. S, **41**, 9 (1998). Presented at The American College of Rheumatology Meeting. San Diego, CA.

Matthew L. Albert, S. Frieda. A. Pearce, Loise M. Francisco, Birthe Sauter, Pampa Roy, Roy L. Silverstein and Nina Bhardwaj. Immature dendritic cells phagocytose apoptotic cells via $\alpha_v\beta_5$ and CD36, and cross-present antigens to CTLs. *Journal of Leukocyte Biology.* Suppl. 2, C8 (1998). The 5th International Congress on Dendritic Cells. Pittsburgh, PA.

Matthew L. Albert, Jong-Il Kim, and Raymond B. Birge. The $\alpha_v\beta_5$ integrin recruits the Crk/Dock180 molecular complex for phagocytosis of apoptotic cells. The Vincent duVigneaud Symposium. Cornell University Medical College, NYC (2000).

Matthew L. Albert, Monique J. Kleijmeer and Nina Bhardwaj. The cross-priming of CD8 T cells via the apoptosis-dependant exogenous pathway requires CD4 help. The 6th International Congress on Dendritic Cells. Port Douglas, Australia (2000).

Matthew L. Albert, Jong-Il Kim, and Raymond B. Birge. The $\alpha_v\beta_5$ integrin recruits the Crk/Dock180 molecular complex for phagocytosis of apoptotic cells. The ELSO Meeting. Geneva, Switzerland (2000).

Matthew L. Albert, Mithila Jegathesan, Robert B. Darnell. Dendritic cell maturation is required for the cross-tolerance of $CD8^+$ T cells. Keystone Symposia on Dendritic Cells—interfaces with immunobiology and medicine. Taos, New Mexico (2001).

Matthew L. Albert and Raymond B. Birge. A Dendritic Cell Restricted Mechanism for the Phagocytosis of Apoptotic Cells. The 7th International Workshop on Langerhans cells. Stressa, Italy (2001).

Mithila Jegathesan, Robert B. Darnell and **Matthew L. Albert**. Skewing antigen cross-presentation toward tolerance. The Annual Meeting of the Academy of Clinical Physicians and Scientisits. New York, NY (2002).

Nathalie Blachére, Robert B. Darnell and **Matthew L. Albert.** Cross-presentation of antigen derived from apoptotic cells occurs in dendritic cells lacking the Transporter associated with Antigen Processing. Presented by N. Blachére at The Antigen Processing and Presentation Meeting, Paris (2002).

Invited Lectures (selected 2000-present)

Vivir La Muerte: dendritic cells cross-present antigen derived from engulfed apoptotic cells. Department of Cell Biology & Institute of Biomembranes. AZU Medical Center, Utrecht, Netherlands. (2000).

Lessons from the Worm: The $\alpha_v\beta_5$ integrin recruits the Crk/Dock180/Rac1 molecular complex for phagocytosis of apoptotic cells. Institut Curie, Paris, France. (2000).

Vivre La Mort: dendritic cells cross-present antigen derived from engulfed apoptotic cells. Institut Cochin de Génétique Moléculaire, Paris, France. (2000).

Vivir La Muerte: dendritic cells cross-present antigen derived from engulfed apoptotic cells. SmithKline Beecham, King of Prussia, PA. (2000).

Mangeant La Mort: The $\alpha_v\beta_5$ integrin recruits the CrkII / DOCK180 molecular switch for phagocytosis of apoptotic cells. Sherring-Plough, Lyon, France. (2000).

Vivir La Muerte: dendritic cells cross-present antigen derived from engulfed apoptotic cells. Society for Leukocyte Biology, Annual Meeting, Boston, MA. (2000).

Vivir La Muerte: The $\alpha_v\beta_5$ integrin recruits the CrkII / DOCK180 / Rac1 molecular complex for phagocytosis of apoptotic cells. Cold Spring Harbor Banbury Workshop on Phagocytosis, Cold Spring Harbor, NY (2000).

Dendritic Cells cross-present antigen derived from apoptotic cells: an exogenous MHC I pathway important for the activation of viral and tumor specific cytotoxic T lymphocytes. The Inflammation Research Association and Pulmonary Research Group, New York (2000).

The Dead can Dance: Defining the cellular and molecular requirements for cross-priming vs. cross-tolerance. Immunobiology Seminar Series. Edinburgh University Medical School, Edinburgh, Scotland (2001).

Resurrecting the Dead: Apoptotic Cells Deliver Antigen to Dendritic Cells for the Activation of Tumor-specific Killer T cells. SUNY Purchase, Purchase NY (2001).

Deciphering the Rosetta Stone of Tumor Immunity—novel approaches to tumor immunotherapy uncovered through the better understanding of the paraneoplastic neurologic disorders. Walter Reed Army Institute of Research, Washington D.C. (2001).

Defining the cellular and molecular requirements for cross-priming vs. cross-tolerance. Immunex Corporation. Seattle, WA (2001).

The Dead can Dance: Defining the cellular and molecular requirements of antigen cross-presentation. National Institute of Health. Bethesda, MD (2001).

Dendritic Cells: The Bridge Between the Innate & Cognate Immunity. Workshop on Innate Immunity—Role in HIV Pathogenesis and Treatment. National Institute of Allergy and Infectious Disease. Gaithersburg, MD (2001).

Antigen Cross-presentation and Tumor Immunity. Department of Biotechnology and Bioscience, University of Milano-Bicocca, Milan (2002).

Tipping the Balance—towards an understanding of antigen cross-presentation. Center for Immunotherapy of Cancer, University of Connecticut, Farmington, CT (2002).

Toward a better understanding of dendritic cell antigen cross-presentation. University of Pittsburgh Cancer Institute, Pittsburgh, PA (2002).

Antigen Cross-presentation and Tumor Immunity. Department of Immunology & Intracellular Parasitism, The Pasteur Institute, Paris (2002).

Antigen Cross-presentation and Tumor Immunity. La Jolla Institute of Allergy and Immunology, La Jolla, CA (2002).

Dendritic Cell Immunobiology. FASEB Summer Research Conference on Transplantation Immunity.Saxton River, Vermont (2002).

Antigen Cross-presentation and Tumor Immunity.Clinical Seminar Series, The Rockefeller University Hospital, New York (2002).

Peptide Epitopes Derived from the Endoplasmic Reticulum of Apoptotic Cells are a Source of Antigen for Cross-presentation. Netherlands Cancer Institute (NKI), Amsterdam (2002).

Tumor Immuniy *versus* Tumor-mediated Immunosupression. Sherring-Plough, Lyon, France. (2003).

Antigen Cross-presentation and BCG-mediated Tumor Immunity in Transition Cell Carcinoma of the Bladder. Urology Oncology Working Group Meeting, New York Hospital, NYC (2003).

Antigen Cross-presentation and Tumor Immunity. Special Seminar, Department of Immunology and Microbiology, The Weill College of Medicine at Cornell University (2003).

Antigen Cross-presentation and HPV pathogenesis. Human Papillomavirus Vaccines—Symposium, Univ of Cambridge, England (2003).

The Role of CD4$^+$ T cells in DC Ac..vation. American Transplant Congress, Washing..on D.C. (2003).

Antigen Cross-presentation and BCG-mediated Tumor Immunity. Hospital Necker, Urology Dept., Paris (2004).

Defining cellular and molecular signals that regulate antigen cross-presentation. Keystone Symposia on Immune Evasion, Taos, NM (2004).

Tumor Immunity & Antigen cross-presentation. Second Military Medical University, Shanghai China (2004).

Death Defying Immunity—apoptotic cells play an active role in antigen cross-presentation. Centre de Recherche INRA. France (2004).

Dendritic cells cross-present antigen from apoptotic cells for immune tolerance. Workshop: Photopheresis and Hematopoetic Stem Cell Transplant—Mechanisms and Clinical Applications. New York (2004).

Direct and cross-presentation of influenza viral antigens by dendritic cells. Departmental Seminar, Virology, Institut Pasteur, Paris (2004).

Apoptosis and Immunity. Keystone Symposia on Survival and Death in Immune Tolerance, Keystone, CO (2005).

Departmental and community service

Faculty search committee, Department of Immunology, Institut Pasteur (2003-2004). Participated in the successful recruitment of Dr. Philippe Bousso.

Organizer of Departmental Seminar Series, Department of Immunology, Institut Pasteur (2004-2005).

co-Organizer of the Annual Congress for the French Society of Immunology. Hosted at Institut Pasteur, a 3 1/2-day congress with 525 participants. (Nov' 2004).

Research Support (past and current)

National Institutes of Health, Medical Scientist Training Grant (1993-2000).

National Cancer Center, Cancer Biology and Tumor Immunology Fellowship (2000-2002).
Title: An animal model for naturally occurring tumor immunity

National Institutes of Health, National Research Service Award (2000-2003).
Title: An animal model for naturally occurring tumor immunity

Burroughs Wellcome Fund, Career Award for Biomedical Science (2001-2003, terminated).
Title: Tumor Immunity versus Tumor-mediated Immunosuppression

National Institutes of Health, NCI—K22 Transitional Career Development Award. (terminated 2003)
Title: Antigen Cross-presentation and Tumor Immunity

Doris Duke Charitable Foundation, Clinical Development Award (2002-2007, transferred to IP).
Title: Tumor Immunity versus Tumor-mediated Immunosuppression

La Ligue Nationale Contre le Cancer (2003-2004)
BCG and Transitional Cell Carcinoma of the Bladder

INSERM—Avenir, AVIN0201 Young Group Leader. (2003-2006)
Title: Antigen Cross-presentation and T cell Immunity

5 year group—Institut Pasteur (2003-2008)
Title: Dendritic Cell Immunobiology

La Ligue Contre le Cancer—Research Program Grant (2004-2006)
Title: Antigen Cross-presentation and T cell Immunity

Patents

Matthew L. Albert, Nina Bhardwaj, Ralph Steinman and Kayo Inaba. Methods for use of Apoptotic Cells to Deliver Antigen to Dendritic Cells for Induction or Tolerization of T cells. *Awarded in part. PCT/US99/03763. WO9942564*

Matthew L. Albert, Nina Bhardwaj and Robert B. Darnell. Methods and Agents for the Detection and Modulation of Cellular Immunity to Immune Privileged Antigens. *Pending. PCT/US99/14827. WO0000825*

Matthew L. Albert and Raymond B. Birge. Genetic Manipulation of Phagocyes for Modulation of Antigen Processing and the Immune Response Therefrom. *Pending. WO0185207*

Matthew L. Albert, Mithila Jegathesan and Robert B. Darnell. Methods for Abrogating a Cellular Immune Response. *Filed as CIP. Pending. WO0185207*

Deborah Braun and **Matthew L. Albert**. Methods for modulating indolamine 2,3 dioxygenase expression and activity. *Filed.*

Apoptotic Cells Deliver Processed Antigen to Dendritic Cells for Cross-Presentation

Nathalie E. Blachère[1], Robert B. Darnell[2,3], Matthew L. Albert[1,4*]

1 Laboratoire d'Immunobiologie des Cellules Dendritiques, Institut Pasteur, Paris, France, 2 The Laboratory of Molecular Neuro-oncology, The Rockefeller University, New York, New York, United States of America, 3 Howard Hughes Medical Institute, The Rockefeller University, New York, New York, United States of America, 4 Institut National de la Santé et de la Recherche Médicale (INSERM) AV0201, Paris, France

Antigen derived from engulfed apoptotic cells can be cross-presented by dendritic cells (DCs) for the generation of major histocompatibility class I/peptide complexes. While the early events of recognition and internalization of the dying cell have been characterized, the antigen-processing pathway or pathways remain unknown. We established a mouse model assaying for the activation of polyclonal T cells reactive to antigen derived from apoptotic cells, and demonstrated two distinct pathways for the trafficking of exogenous epitopes. In the first, exogenous antigen is dependent on the DC's expression of a functional transporter associated with antigen processing (TAP). Surprisingly, we found evidence that a second pathway exists in which transfer of processed antigen from the dying cell allows formation of major histocompatibility class I/peptide complexes in TAP-deficient DCs. In vivo data suggest that in situations of stress (e.g., viral infection), this latter pathway may be more efficient, illustrating that dying cells may preselect immunologically important antigenic determinants.

Citation: Blachère NE, Darnell RB, Albert ML (2005) Apoptotic cells deliver processed antigen to dendritic cells for cross-presentation. PLoS Biol 3(6): e185.

Introduction

Apoptosis is considered the primary means by which physiologic cell death occurs [1]. The fate of apoptotic material is rapid clearance and degradation by phagocytes. There is, however, growing evidence that apoptotic death need not be an endpoint, and that dying cells are capable of transferring antigen to the immune system for the induction of T cell immunity [2,3]. We have previously demonstrated that human dendritic cells (DCs) phagocytose apoptotic cells, and rather than degrading the internalized material, the DCs are capable of generating peptide epitopes for major histocompatibility (MHC) I molecules and activating viral- and tumor-antigen-specific $CD8^+$ T cells [4–6]. This pathway has been referred to as cross-presentation for its ability to "cross" classically defined restrictions for MHC I antigen presentation [7]. Our work has offered a physiologically relevant mechanism for the in vivo phenomenon of cross-presentation, which accounts for both the cross-priming and cross-tolerization of tissue-restricted antigen-specific $CD8^+$ T cells [8–10]. We have demonstrated that antigen capture occurs via receptor-mediated phagocytosis [5,11], and that internalized apoptotic material can be located within the MHC II–containing compartment [12]; however, the trafficking of antigen from the apoptotic cell to the DC for generation of MHC I/peptide (MHC I/pep) complexes has not been fully characterized.

To define the cellular machinery required for cross-presentation, several studies have focused on the use of cells expressing vector-encoded gene products to test whether proteasomal substrates (e.g., intact proteins) or chaperoned peptides serve as the source of antigen [13–15]. While these studies conclude that cellular proteins are the major source of antigen transferred to the immune system, the inability to demonstrate transfer of cell-associated antigen, or the lack of processed antigen within the dying cell, may have skewed the observed results. In other studies, the use of exogenous antigen bound to latex beads [16,17], derived from internalized immune complexes [18] or whole protein [19], does not permit antigen processing to occur prior to capture by an antigen-presenting cell (APC); therefore, it is not surprising that these models demonstrate that the phagosome-to-cytosol pathway is the dominant means by which antigen is trafficked for processing and presentation onto MHC I.

To examine whether antigen processed within the dying cell can be transferred to the DC, we designed in vitro and in vivo experiments to track the activation of polyclonal influenza-reactive T cell responses stimulated by DCs cross-presenting antigen from haplotype-mismatched apoptotic cells. We report the utilization of two independent pathways by which internalized antigen may access MHC I within the DC: in one, the substrate for cross-presentation is whole or partially degraded protein, which must be further processed by the DC; and in the other, we find evidence for processed antigen accessing the MHC I pathway of the DC. Concerning the in vivo presentation of viral antigen, this latter pathway seems dominant, thus permitting efficient loading of MHC I/pep complexes by the DC.

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Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; ER, endoplasmic reticulum; HAU, hemaglutanin units; HSP, heat shock protein; IFN-γ, interferon-γ; IL-12, interleukin-12; MHC, major histocompatibility; mAb, monoclonal antibody; MHC I/pep, major histocompatibility class I/peptide; NP, A/PR/8 nucleoprotein; PA, A/PR/8 acid polymerase; WT, wild-type

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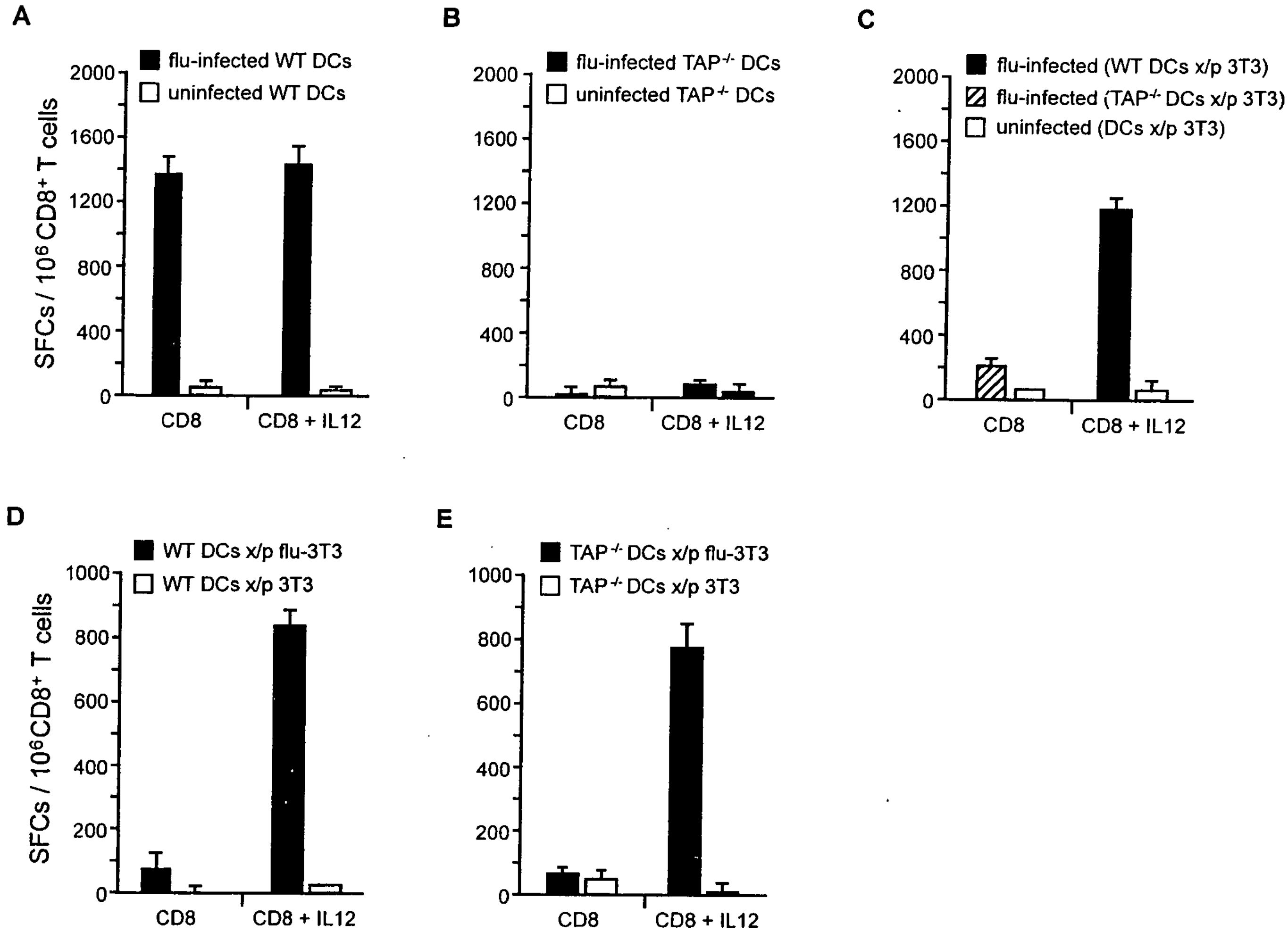

Figure 1. $TAP^{-/-}$ DCs Cross-Present Antigen Derived from Apoptotic Cells

(A and B) Antigen presentation via the endogenous pathway was evaluated in WT DCs and $TAP^{-/-}$ DCs by directly infecting cells with influenza virus and assaying for T cell activation. IFN-γ production and T cell precursor frequency were determined using an ELISPOT assay.
(C) To evaluate transfer of TAP activity from the dying cells to DCs, WT or $TAP^{-/-}$ DCs after capture of apoptotic cells were directly infected and tested for their respective ability to activate $CD8^+$ T cells via the endogenous pathway.
(D and E) WT DCs (D) or $TAP^{-/-}$ DCs (E) were co-cultured for 36–48 h with influenza-infected or uninfected allogeneic cells in the presence of TNF-α. As above, mature DCs were harvested and assayed for their ability to stimulate influenza-reactive $CD8^+$ T cells. To bypass the requirement for $CD4^+$ T cell help in the activation of $CD8^+$ T cells via the exogenous pathway, IL-12 was added to the cultures.
Spot-forming cells (SFCs) per 10^6 T cells are reported. Data are representative of three experiments. Values are averages of triplicate wells with error bars indicating standard deviation.
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Results

In order to dissect the pathway or pathways by which antigens derived from apoptotic cells are processed and presented by DCs, we established in vivo and in vitro systems that permit monitoring of direct presentation and cross-presentation of antigen (Protocol S1; Figure S1). Using these model systems, we tested the hypothesis that dying cells participate in the processing of antigen for cross-presentation. To restrict the DCs' capacity to process antigen, we employed bone-marrow-derived DCs prepared from mice deficient in TAP-1. The generation of antigen-specific MHC I/pep complexes was assayed based on the ability to stimulate influenza-reactive T cells. In all experiments, interleukin-12 (IL-12) was added to the DC/$CD8^+$ T cell cultures to bypass the requirement for $CD4^+$ T cell help [8].

To ensure that the influenza antigens being monitored required transporter activity for the generation of MHC I/pep complexes, we directly infected DCs prepared from $TAP^{-/-}$ mice (Figure 1). As has previously been reported, no T cell activation was evident when infected $TAP^{-/-}$ DCs were employed (Figure 1A and 1B). Furthermore, we established that the $TAP^{-/-}$ DCs efficiently engulfed apoptotic bodies and that the kinetics of uptake were similar to those evident in wild-type (WT) DCs (Figure S2). DCs that had internalized dying cells were tested for their ability to cross-present antigen; in contrast to their ability to present antigen via the "classical" MHC I pathway, the $TAP^{-/-}$ DCs were able to cross-present influenza antigen derived from MHC-mismatched apoptotic cells as efficiently as WT DCs (Figure 1D and 1E). To rule out the possibility that transporter activity was simply being transferred via fusion of membranes between the

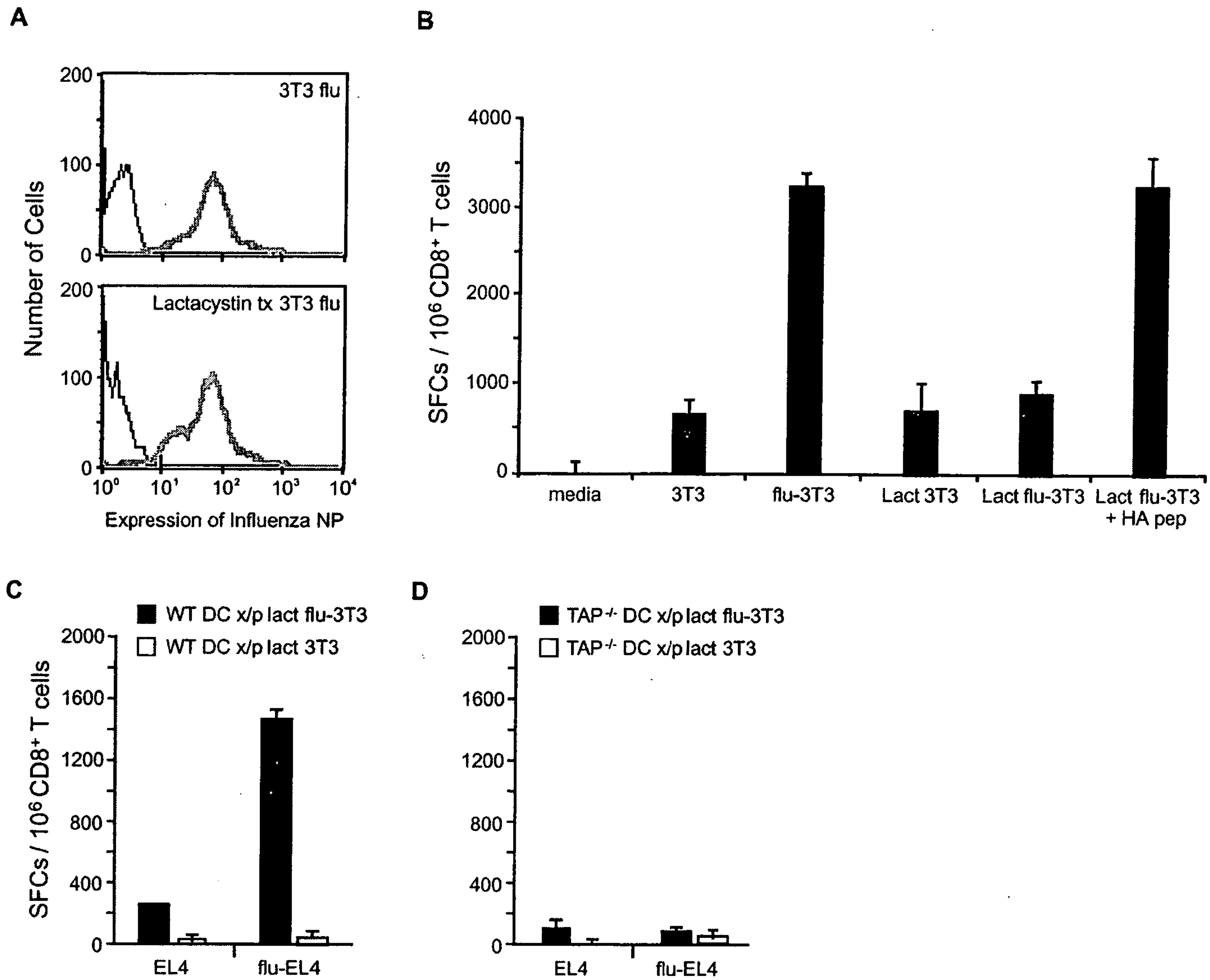

Figure 2. Processed Antigen from the Dying Cell Is Required for MHC I Presentation in TAP$^{-/-}$ DCs

To generate apoptotic cells lacking processed antigen, lactacystin pretreatment of influenza-infected H-2^{d} 3T3 cells was performed. Expression of influenza antigen was evaluated by intracellular FACS analysis using influenza NP mAbs followed by PE-conjugated goat anti-mouse mAb (A). Expression of MHC I/pep complexes in the lactacystin-treated 3T3 cells was evaluated by monitoring the activation of H-2^{d}-restricted influenza hemagglutanin-reactive T cells. The K^{d}-restricted immunodominant peptide (HA$_{210-219}$) derived from hemagglutanin was pulsed onto 3T3 cells and served as a positive control (B). The influenza-infected H-2^{d} 3T3 cells were then induced to undergo apoptosis, and co-cultures were generated using C57BL/6 WT DCs (C) or TAP$^{-/-}$ DCs (D). To evaluate T cell activation and expansion, DCs cross-presenting antigen were cultured with CD8^{+} T cells in the presence of IL-12 for 7–8 d. T cells were then harvested and tested for influenza reactivity in a 20-h IFN-γ ELISPOT. H-2^{b} EL4 cells with or without influenza infection served as the stimulators in the ELISPOT assay as above. Data are representative of two experiments. Values are averages of triplicate wells with error bars indicating standard deviation.

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apoptotic cells and the DC during phagocytosis, we tested whether TAP$^{-/-}$ DCs that had previously internalized uninfected TAP-expressing apoptotic cells could now present antigen after direct infection with influenza (flu-[TAP$^{-/-}$ DC x/p 3T3]; Figure 1C). We detected background levels of T cell activation in these assays as compared to the robust stimulation observed using influenza-infected WT DCs (Figure 1C), arguing against such a mechanism and supporting the possibility that TAP$^{-/-}$ DCs are capturing processed antigen from apoptotic cells.

To further establish the role for antigen processing in the dying cell, we generated apoptotic cells that expressed influenza proteins but were unable to process the influenza viral antigens. Prior to infection with influenza and the induction of apoptosis, 3T3 cells were treated with the proteasome inhibitor lactacystin [20]. We found that the lactacystin-treated cells expressed levels of influenza antigen similar to untreated cells (Figure 2A). The inhibition of proteasome activity was confirmed functionally using treated cells as stimulators for HA-reactive T cells restricted to H-2^{d}, the MHC haplotype of the dying 3T3 cells (Figure 2B). Parallel cultures were triggered to undergo apoptosis and were co-cultured with WT or TAP$^{-/-}$ DCs as described in the Materials and Methods. Cross-presentation of antigen by the DCs was evaluated based on the activation of influenza-reactive CD8^{+} T cells. We found that the lactacystin-treated apoptotic cells

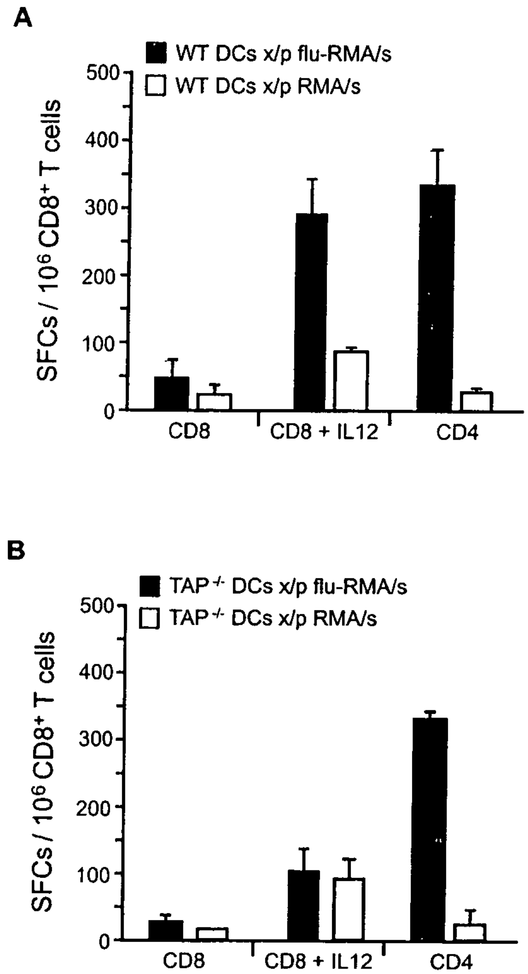

Figure 3. Transporter Activity Is Required in Either the Apoptotic Cell or the DC for Efficient Cross-Presentation of Antigen

RMA/S cells were infected with influenza and irradiated with UVB to allow for antigen loading and the induction of apoptotic death. Co-cultures were generated as described in the Materials and Methods using WT DCs (A) or $TAP^{-/-}$ DCs (B). DCs were harvested and tested for their ability to cross-present antigen and activate influenza-reactive $CD8^+$ T cells as measured in a 40-h ELISPOT assay. To evaluate loading of MHC II, WT DCs and $TAP^{-/-}$DCs that had captured apoptotic antigen were tested for their ability to activate influenza-reactive CD4+ T cells. Data are representative of four experiments. Values are averages of triplicate wells with error bars indicating standard deviation.

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were competent to serve as a source of antigen for WT DCs (Figure 2C); however, the lack of processed antigen in lactacystin-treated 3T3 cells prevented cross-presentation by $TAP^{-/-}$ DCs (Figure 2D). This result further establishes that transporter activity is not passed from the dying cell to the $TAP^{-/-}$ DC as a result of an ill-defined fusion event. Instead, it is an active process whereby processed antigen within the dying cell is being utilized by the DC for the generation of MHC I/pep complexes.

To determine the importance of antigen access to the dying cell's endoplasmic reticulum (ER), we used RMA/s cells, which are deficient in TAP-2, as a source of influenza antigen. These cells express influenza proteins upon infection (Figure S3A), but do not facilitate peptide transport into the ER, as established by their inability to re-stimulate an influenza-

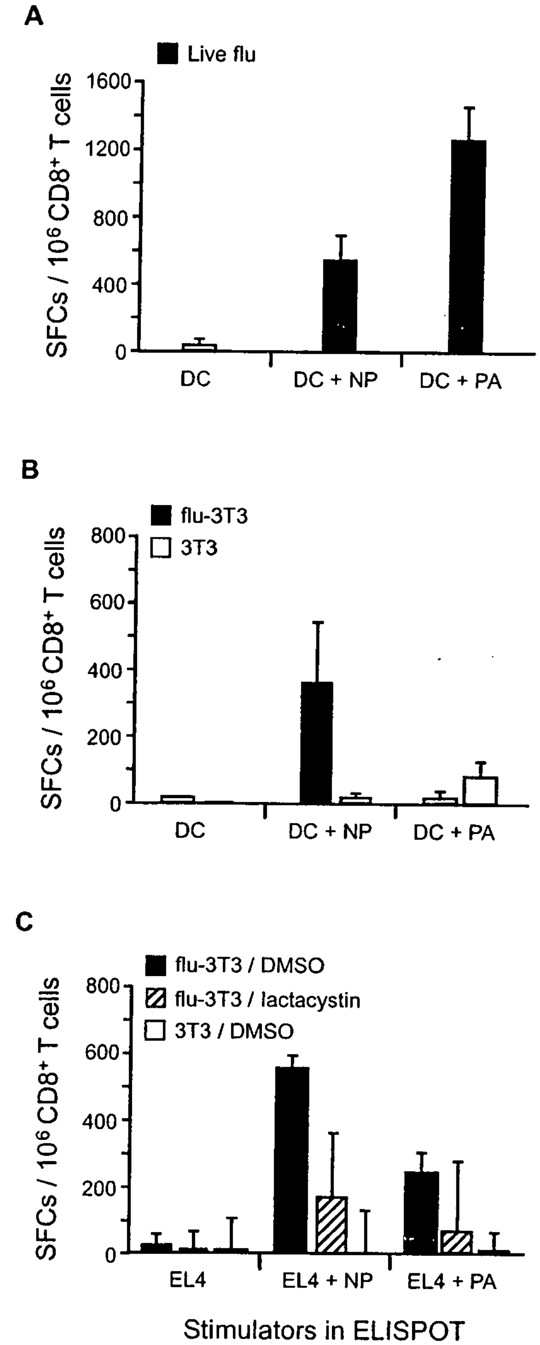

Figure 4. Immunization with Apoptotic Cells Results in the Selective Priming of T Cells Reactive to Processed Antigen

(A and B) C57BL/6 mice were immunized intraperitoneally with 300 HAU of influenza (A), or 5×10^6 infected apoptotic 3T3 cells (B). After 14 d, splenocytes were harvested, and $CD8^+$ T cells were purified. To assay for the specificity of these cells, an IFN-γ ELISPOT was performed using the following stimulators: DCs alone or DCs pulsed with either 1 μM $NP_{366-374}$ or 1 μM $PA_{224-233}$ peptide.

(C) C57BL/6 mice were immunized intraperitoneally with 5×10^6 untreated versus lactacystin-treated influenza-infected apoptotic 3T3 cells. As above, 14 d after priming, splenocytes were harvested, and $CD8^+$ T cells were purified and assayed for their reactivity to $NP_{366-374}$ versus $PA_{224-233}$. In this experiment, peptide-pulsed EL4 cells were employed as the stimulators. Data are representative of two experiments. Values are averages of triplicate wells with error bars indicating standard deviation.

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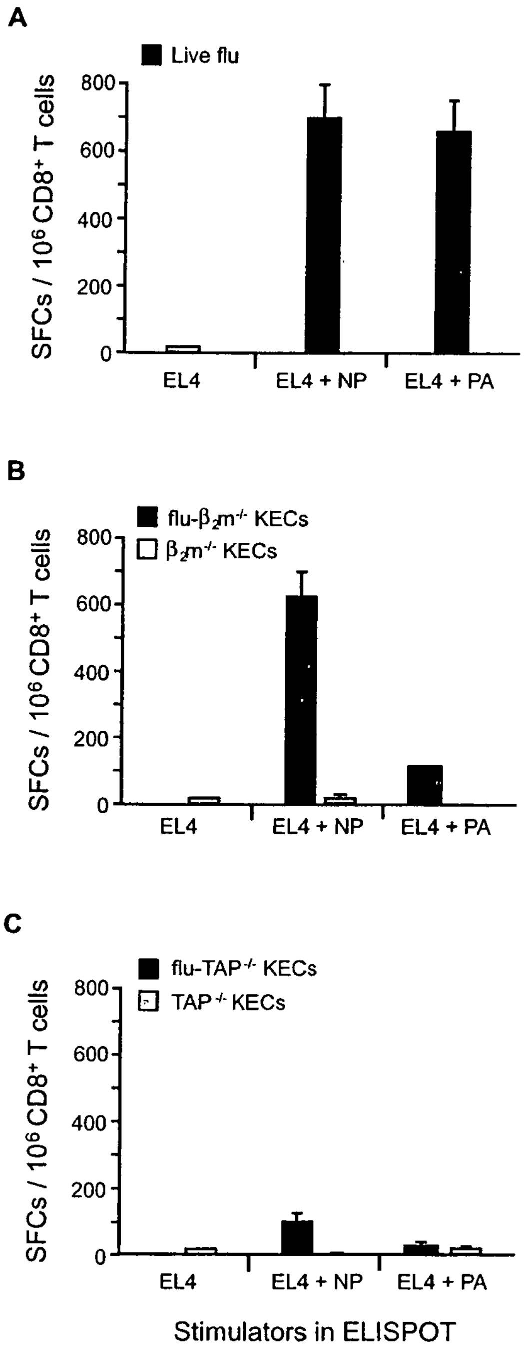

Figure 5. Processed Antigen within the Dying Cell Is Required for Efficient In Vivo Priming

C57BL/6 mice were immunized intraperitoneally with 300 HAU of influenza (A), or 2×10^6 infected apoptotic kidney epithelial cells derived from β_2m-deficient (B) or TAP-deficient mice (C). After 14 d, splenocytes were harvested, and CD8$^+$ T cells were purified. To assay for the specificity of these cells, an IFN-γ ELISPOT was performed using the following stimulators: EL4 cells alone or EL4 cells pulsed with either 1 μM $NP_{366-374}$ or 1 μM $PA_{224-233}$ peptide. Values are averages of triplicate wells with error bars indicating standard deviation.

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reactive CD8$^+$ T cell line (Figure S3B). When WT DCs were employed as the APC, RMA/s served as a source of antigen for the generation of MHC I/pep complexes (Figure 3A); in contrast, when TAP$^{-/-}$ DCs were used, no activation of CD8$^+$ T cells was observed (Figure 3B). While the absence of TAP in both the dying cell and the DC prevented loading of MHC I, antigen presentation on MHC II was unaffected, as equivalent stimulation of influenza-reactive CD4$^+$ T cells was observed when using WT or TAP$^{-/-}$ DCs (Figure 3A and 3B).

Together, these results suggest the presence of two independent pathways by which antigen may be cross-presented. The first is a pathway that requires transporter activity in the DC—presumably relying on the transport of exogenous antigen from the phagosome to the cytosol—and accounts for the requirement for TAP-sufficient DCs to generate MHC I/pep complexes in conditions where the peptide is derived from whole or partially degraded protein that is present within internalized apoptotic lactacystin-treated 3T3 cells (see Figure 2C) or TAP$^{-/-}$ RMA/S cells (see Figure 3B). In the second, the DCs are able to capture processed antigen present within proteasome- and TAP-competent dying cells; notably, this latter antigen source may be cross-presented without a need for further transporter activity within the DC (see Figure 1E).

To evaluate the in vivo relevance of these findings, we took advantage of a recent observation made by Woodland and colleagues regarding the unique ability of DCs to generate MHC I peptides derived from the influenza A/PR/8 acid polymerase (PA) protein [21]. They demonstrated that while most cells are capable of processing influenza A/PR/8 nucleoprotein (NP), only DCs process and present the epitope $PA_{224-233}$. We confirmed this result and demonstrated that both epitopes required transporter activity in the infected DC—in other words, influenza-infected TAP$^{-/-}$ DCs activated neither $NP_{366-374}$- nor $PA_{224-233}$-specific T cells (data not shown).

We next analyzed the T cell repertoire generated after priming C57BL/6 (H-2^b) mice with live influenza versus influenza-infected apoptotic 3T3 (H-2^d) cells. Given that the apoptotic cells express both NP and PA protein, but process only $NP_{366-374}$, the in vivo activation of $NP_{366-374}$- and $PA_{224-233}$-specific T cells would suggest that the DC was responsible for processing the antigen, whereas a response to $NP_{366-374}$ in the absence of a response to $PA_{224-233}$ would imply that processed antigen was the preferred source of antigen for cross-presentation. As reported by Crowe et al. [21], priming the mice with 300 hemaglutanin units (HAU) of live influenza resulted in the activation of both $NP_{366-374}$- and $PA_{224-233}$-reactive T cells (Figure 4A). However, when apoptotic influenza-infected 3T3 cells were injected into naïve mice, we observed a robust $NP_{366-374}$-specific response and only weak reactivity to $PA_{224-233}$ (Figure 4B). To examine whether the response was indeed due to the processing of antigen within the dying cell, we primed naïve mice using lactacystin-treated influenza-infected 3T3 cells (prepared as above), and observed a marked reduction in the efficiency of $NP_{366-374}$-reactive T cell priming (Figure 4C). Similarly, we demonstrated that infected kidney epithelial cells derived from β_2m-deficient but not TAP$^{-/-}$ mice were capable of priming $NP_{366-374}$-specific T cells (Figure 5). These data again highlight the importance of processed antigen within the ER of the dying cell. In sum, we demonstrated the in vivo relevance of DCs capturing processed antigen derived from dying cells for the cross-priming of CD8$^+$ T cells, and suggest that in some experimental conditions, this pathway may be more efficient than the cross-priming of whole protein.

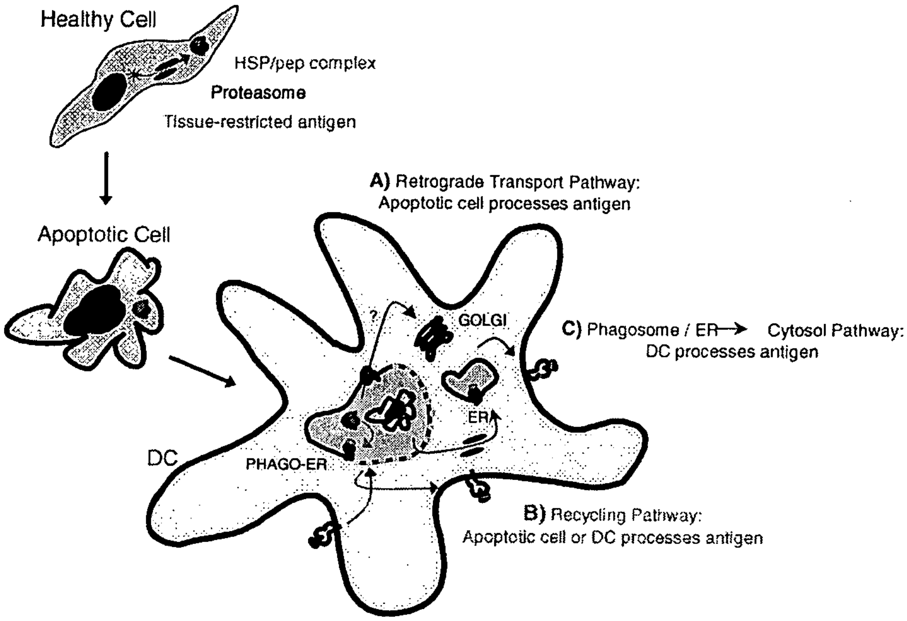

Figure 6. An Active Role for Apoptotic Cells in the Transfer of Antigen to DCs

We propose that apoptotic cells play an active role through the transfer of processed antigen to DCs for the generation of MHC I/pep complexes. This pathway may be dominant in the presentation of infectious antigen as the virus may co-opt cellular translational machinery, resulting in high levels of viral protein, and the upregulation of stress proteins, as well as inducing apoptotic cell death. Defective ribosomal initiation products chaperoned by HSPs offer a potential source of antigen. Within the DC, HSPs derived from the internalized apoptotic cell may traffic via a retrograde transport pathway, shuttled to the *trans*-Golgi and then the ER via binding to KDEL receptors (A). Alternatively, the evidence for phagosome–ER (PHAGO-ER) fusion and/or the recycling of MHC I from the plasma membrane offers the possibility that processed antigen may interact directly with the DC's MHC I (B). As ER chaperones within the phagocytosed cell would be bound to the pool of peptides derived from newly synthesized proteins, these pathways offer the DC an accurate representation of what occurred immediately prior to death (A and B). At high concentrations of protein, we also find evidence for the DC processing the cross-presented antigen. This likely occurs via a phago–ER-to-cytosol pathway as has been previously described (C).

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Discussion

Several models for antigen cross-presentation have evaluated a role for transporter activity, and most report that COOH terminal processing by the proteasome and utilization of TAP by the APC is essential [22,23]. This is understandable when exogenous antigen is derived from internalized immune complexes [18], antigen-coated latex beads [16,17], or whole protein [19]. In these instances, there is no ability for antigen processing to occur in a manner that would permit loading of DC MHC I in the absence of transporter activity. With respect to in vitro systems that have reported a TAP-independent pathway, the antigens successfully presented seem limited to peptides immediately COOH-terminal to an ER targeting sequence, or those within secreted or transmembrane proteins that are processed by still undefined ER proteases [24,25]. It has also been demonstrated that at high levels of antigen challenge, it is possible for peptide epitopes to be generated by Cathepsin S within the phagolysosome [26].

In this study, we restricted our analysis to physiologically relevant levels of antigen (all of which require proteasome processing and TAP activity), and asked whether dying cells serve as a source of whole protein or whether they may also participate in antigen presentation by generating processed antigen that may be transferred to DCs. As has been previously shown, our data demonstrated that WT DCs can process antigen from cells that contain whole or partially processed protein (see Figures 2C and 3A). To assess the ability of the dying cell to process the antigen, $TAP^{-/-}$ DCs were used. Applying this strategy, we identified the existence of an antigen cross-presentation pathway that utilizes proteasome and transporter activity present in the dying cell (see Figures 1, 2, and 3). Importantly, when lactacystin-treated or $TAP^{-/-}$ apoptotic cells were the source of antigen, the $TAP^{-/-}$ DCs were no longer capable of cross-presenting antigen (see Figures 2D and 3B). Furthermore, we demonstrated activation of $CD4^{+}$ T cells in all experimental conditions, illustrating that DCs indeed captured dying cells expressing influenza antigen, even in situations where MHC I presentation was inhibited. While this in vitro system allowed us to carefully control the nature of the antigen present in the dying cells (whole protein or processed antigen) and permitted us to ensure the transfer of cell-associated protein to the DCs, we also tested the ability to prime T cells in vivo. Taking advantage of the differential processing of influenza antigen by DCs versus other cell types, we demonstrated that in situations of direct infection, DCs processed the antigen. When dying cells were the source of antigen, we observed a preferential skewing of T cell cross-priming toward the protein that could be processed by the apoptotic cell. Lactacystin treatment of the influenza-infected cell or the use of $TAP^{-/-}$ cells confirmed the requirement for proteasome processing and transporter activity within the dying cell (see Figures 4 and 5). Furthermore, the ability to cross-prime influenza-specific T cells with β_2m-deficient but not TAP-deficient cells indicated that the antigen was originating from the ER of the dying cell (see Figure 5). When greater numbers

of apoptotic cells were used (10–50×), it was possible to observe cross-priming of whole or partially degraded protein (data not shown). Our findings support the in vitro work shown here and that of Serna et al. [27]—cross-priming of influenza antigen favors the processed antigen within the dying cell (Figure 6). Indeed, apoptotic cells may play an active role in antigen presentation through the delivery of processed antigen, in turn allowing for efficient generation of MHC I/pep complexes by the DC.

The identification of this pathway raises the intriguing possibility that ER chaperones within the apoptotic cell are facilitating delivery of peptide epitopes to the DC (see Figure 6). Notably, the heat shock proteins (HSPs) GP96 and calreticulin have been shown to associate with newly processed cytosolic-derived epitopes, and when injected in vivo, they cross-prime cytotoxic T lymphocytes [28–30]. The recent reports of ER-phagosome fusion [31] suggest that HSP/peptide complexes may be capable of direct interaction with the DC's MHC I; alternatively, HSPs containing a KDEL motif (Lys-Asp-Glu-Leu) may employ a retrograde transport pathway to directly access the ER [24]. As ER chaperones within the phagocytosed cell would be bound to the pool of peptides derived from newly synthesized proteins [32], they offer the DC an accurate representation of what was being translated immediately prior to death. As an interesting alternative to HSPs, the processed antigen transferred may be the pool of peptides bound to chromatin [33]. As the nucleus lacks efficient peptidase activity, antigen may be protected within the nuclear remnants of an apoptotic body. If this were occurring, we predict that loading of the MHC I in the DC would occur in the phagolysosome.

Our findings are of particular interest when placed in the context of three recent studies that report that cell-associated whole protein is the primary source of antigen for cross-priming [13–15]. We fully appreciate that our study may reflect the choice of a viral model for cross-priming and acknowledge that the experimental details will influence the conclusions. In this light, it is important to consider the differences between the chosen model systems. In the work of Shen and Rock [13], lysates prepared from ovalbumin-transfected cell lines were used as a source of antigen, testing different subcellular fractions for their ability to prime animals. This study argues that intact cellular protein, rather than peptides or HSP/peptide complexes, is the main source of antigen for cross-presentation. Considering their use of nitrogen cavitation as the method for disrupting cells, which has been reported to dissociate antigenic peptides from HSP70 and GP96 [34,35], it would be expected that the HSPs within their lysates would indeed be inert. As a result, this model may have been biased toward the cross-presentation of whole antigen. Wolkers et al. [15] demonstrated that peptides present in the secretory domain of nascent proteins are not efficiently cross-presented, while the stable epitope within the mature protein is indeed transferred to APCs. The in vivo studies presented seem to be tracking the cross-presentation of secreted protein, not cell-associated protein. Use of H-2^d × H-2^b F$_1$ mice to demonstrate cross-presentation from P815 cells supports the requirement for the P815 (H-2^d) to remain alive. An alternate interpretation of their experiments is that soluble proteins produced (in large quantities) by growing tumors resulted in the observed in vivo T cell activation. As a result, there may have been little opportunity for processed cell-associated protein to gain access to a DC. Finally, the study from Norbury et al. [14] reported that proteasome substrates (rather than peptides) are critical for achieving antigen transfer for cross-presentation. These studies rely heavily on the use of lactacystin to inhibit proteasome activity. However, while they show the persistence of whole protein, they do not demonstrate that lactacystin prevents the generation of processed peptides in the experimental models used. In the studies described herein, 100 μM lactacystin with a maintenance dose of 1 μM, to inhibit newly synthesized proteasomes (during infection and antigen expression), is required to block the generation of peptides secondary to influenza infection. Given the possibility of newly synthesized proteasomes and/or proteasome-independent processing acting on their artificial constructs for the generation of peptide epitopes, it is critical that functional studies be used to exclude the production of small amounts of processed antigen.

In sum, while cell-associated whole protein is important in cross-priming, previous studies have not excluded proteasome products or HSP/peptide complexes as substrates for in vivo cross-priming. As shown here, the apoptotic cell may in fact play a critical role in processing antigen for cross-presentation, in essence preselecting immunologically important antigenic determinants. A comprehensive model accounting for antigen derived from whole protein as well as processed antigens from apoptotic cells is needed to more clearly define the pathways of antigen cross-priming in physiologic (resting) as well as pathologic (stress) situations.

Materials and Methods

Mice. WT and TAP-1-deficient C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine, United States). In all experiments, 4- to 6-wk-old female mice were employed.

Antibodies, cell lines, and reagents. All FACS antibodies used in this study were obtained from BD Biosciences Pharmingen (San Diego, California, United States); reagents for the ELISPOT assays were obtained from Mabtech (Stockholm, Sweden). PC3 cells, a human prostate cancer cell line, were obtained from American Type Culture Collection (ATCC) (Manassas, Virginia, United States). BALB/3T3 cells clone A31 (3T3) were obtained from ATCC. RMA/S, a TAP-deficient T cell lymphoma cell line derived from the Rauscher murine leukemia virus–induced RBL-5 cell line, was employed [36]. β_2m- and TAP-deficient kidney epithelial cells were derived from organ culture followed by a 2-wk in vitro expansion. All cell lines were grown in DMEM containing 10% fetal bovine serum, supplemented with nonessential amino acids, sodium pyruvate, glutamine, 2β-mercaptoethanol, and gentamicin (D-10). Human influenza A/PR/8 was provided as allantoic fluid from Charles River Laboratories (Wilmington, Massachusetts, United States) and used at a 1:3 dilution to infect PC3, 3T3, or RMA/S cells (1,000 HAU/10^6 cells) or 1:10 dilution to infect DCs (300 HAU/10^6 cells). Recombinant mouse TNF-α and IL-12 were obtained from R&D Systems (Minneapolis, Minnesota, United States).

Preparation of antigen-loaded DCs. Bone-marrow-derived DCs were prepared as previously described [37]. In brief, bone marrow obtained from tibia and femurs was lysed of red blood cells and cultured at a density of 3 × 10^6 cells/well in six-well plates with RPMI containing 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, glutamine, 2β-mercaptoethanol, gentamicin (R-10), and in the presence of GM-CSF (provided by J558L cells transduced with GM-CSF, used 3% vol/vol). Fresh GM-CSF-supplemented medium was added to the wells on days 2, 4, and 6. On day 7, DCs were harvested and plated in fresh wells with or without apoptotic cells. In addition, a maturation stimulus, 80 ng/ml rmTNF-α, was added. To generate influenza-infected apoptotic cells, living cells were first infected with influenza for 1 h at 37 °C in serum-free medium. To allow for expression of viral proteins, 3–5 × 10^6 infected cells per well of a six-well plate were cultured for 5 h at 37 °C. Cells were washed three

times with 3 ml of PBS and were UVB irradiated (120 mJ/cm^2) in 0.5 ml of PBS, and 0.5 ml of R-10 was added. Cells were allowed to undergo apoptosis for 8–10 h prior to adding 10^6 immature DCs. Non-adherent cells were harvested 36 h later, and mature DCs were purified to greater than 95% purity using anti-CD11c microbeads and LS$^+$ columns (Miltenyi Biotec, Bergisch Gladbach, Germany). DCs were monitored by FACS and found to express high levels of I-A^b and CD40. To generate influenza-infected DCs, day 9 mature DCs were infected with influenza for 1 h at 37 °C in serum-free medium. These cells were washed three times in serum containing medium, counted, and used directly.

In vitro cross-presentation studies. Four- to six-week-old mice were infected intraperitoneally with 200–300 HAU of influenza A/PR/8–1976 (Charles River, North Franklin, Connecticut, United States). After 2–4 wk, CD4$^+$ and CD8$^+$ T cells were isolated using MACS purification (Miltenyi Biotec). These cells served as responders in antigen cross-presentation ELISPOT assays. Stimulators included WT or TAP$^{-/-}$ DCs presenting antigen via the endogenous or exogenous pathway. To achieve a ratio of 30 T cells to one DC, 2×10^5 T cells were added to 6.6×10^3 DCs. Cultures were incubated in the plates for 20–36 h at 37 °C, after which cells were washed out of the ELISPOT plates using a mild detergent followed by incubation with 1 μg/ml biotin-conjugated α-interferon-γ (α-IFN-γ) monoclonal antibody (mAb) (BD Biosciences Pharmingen, clone XMG1.2). Wells were then developed using the Vectastain Elite Kit as per manufacturer's instructions (Vector Laboratories, Burlingame, California, United States). Colored spots represent IFN-γ-releasing cells and are reported as spot-forming cells per 10^6 cells. The ELISPOT plate evaluation was performed in a blinded fashion by an independent evaluation service (ZellNet Consulting, Fort Lee, New Jersey, United States) using an automated ELISPOT reader (Carl Zeiss, Thornwood, New York, United States) with KS Elispot 4.3 software.

In vivo cross-priming studies. Mice were primed intraperitoneally using influenza A/PR/8-infected apoptotic cells. Ten to 14 d post-immunization, spleens were harvested and CD8$^+$ T cells were isolated using MACS purification (Miltenyi Biotec). A total of 2×10^5 T cells were added to 2×10^4 peptide-pulsed DCs or EL4 cells (haplotype-matched cell line) in the ELISPOT plates, pre-coated with 5 μg/ml of a primary α-IFN-γ mAb (Mabtech, clone AN18). Cultures were incubated for 20–36 h at 37 °C, and developed as above.

Supporting Information

Figure S1. DC Cross-Presenting Apoptotic Cells Prime CD8$^+$ T Cells In Vivo and Serve As Targets for Effector CD8$^+$ T Cells In Vitro

(A) C57BL/6 mice were immunized intra-footpad with 10^5 DCs cross-presenting apoptotic, influenza-infected PC3 cells, or apoptotic influenza-infected PC3 cells alone. Six days following this single immunization, draining lymph nodes were harvested, CD8$^+$ T cells were immediately purified and tested for their ability to respond to syngeneic stimulator cells with or without antigen in a 20-hr IFN-γ ELISPOT assay. SFCs per 10^6 CD8+ T cells are reported.
(B) As shown in the schematic representation, surface expression of influenza-peptide-loaded MHC I by DCs was monitored using a modified cytotoxicity assay. After charging the DCs with antigen via the endogenous or exogenous pathways, they were loaded with ^{51}Cr and used as targets for previously activated influenza-specific cytotoxic T lymphocytes. This assay is designed to evaluate the surface expression of MHC I/pep complexes. DCs were loaded via the exogenous pathway with influenza-infected allogeneic 3T3 cells (filled black squares) or uninfected 3T3 cells (open black squares). Alternatively, DCs were directly infected, thus presenting antigen via the endogenous pathway (filled red squares) or left uninfected (open red squares). After antigen expression or capture of the apoptotic material, respectively, DCs were loaded with ^{51}Cr and tested as targets. After 5 h, supernatants were collected and percent cytotoxicity was calculated. Percent cytotoxicity = (experimental-well ^{51}Cr release − spontaneous release)/(total release − spontaneous release) × 100.

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Figure S2. TAP$^{-/-}$ DCs Efficiently Phagocytose Apoptotic Cells

Immature DCs from C57BL/6 WT or TAP-1-deficient (TAP$^{-/-}$) mice were prepared as above and labeled with the PKH-67-GL (green) fluorescent cell linker (A and B). These cells were next added to PKH-26-GL-labeled (red) apoptotic cells (ACs) for 7 h at a ratio of one DC to five apoptotic cells in the presence or absence of EDTA. FACS Calibur analysis allowed for the detection of double-positive cells (A), indicating that the green DCs captured red apoptotic material. Phagocytosis was calculated as the percent double-positive cells per total population of DCs (A). Samples of DCs alone and apoptotic cells alone were used for setting the parameters of the flow cytometer. The kinetics of phagocytosis were monitored throughout the experiment, and the percent double-positive cells is reported (B).

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Figure S3. RMA/s Cells Express but Do Not Present Influenza Antigen on MHC I

(A) RMA or RMA/S cells were infected with influenza and incubated at 37 °C for 5 h to allow for viral antigen expression. Cells were fixed, permeabilized, and stained using NP mAbs followed by PE-conjugated goat anti-mouse mAb. Analysis was performed on a FACS Calibur, and histograms are shown.
(B) Infected RMA or RMA/s cells were used as stimulators in an ELISPOT assay, testing for their ability to stimulate influenza-reactive CD8$^+$ T cells. The influenza A/PR/8 $NP_{366-374}$ peptide restricted for D^b was pulsed onto RMA or RMA/s cells and served as a positive control. Values are averages of triplicate wells with error bars indicating standard deviation.

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Protocol S1. DCs Capture Apoptotic Cells and Cross-Present Antigen to CD8$^+$ T Cells

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Accession Numbers

The NCBI Entrez Protein (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = Protein) accession numbers for the gene products discussed in this paper are influenza PA (AAA43619), influenza NP (B36754), and K^d-restricted immunodominant peptide ($HA_{210-219}$) derived from hemagglutanin (NP040980).

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Author contributions. NEB, RBD, and MLA conceived and designed the experiments. NEB performed the experiments. NEB, RBD, and MLA analyzed the data. RBD and MLA contributed reagents/materials/analysis tools. MLA wrote the paper.

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